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# Uptake of Selenite by *Saccharomyces cerevisiae* Involves the High and Low Affinity Orthophosphate Transporters

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**Myriam Lazard**[‡§1], **Sylvain Blanquet**[‡§], **Paola Fisicaro**[¶], **Guillaume Labarraque**[¶], **and Pierre Plateau**[‡§]

*From the* [‡]*Laboratoire de Biochimie and* [§]*CNRS UMR7654, Laboratoire de Biochimie, Ecole Polytechnique, F-91128 Palaiseau Cedex, France and the* [¶]*Department of Biomedical and Inorganic Chemistry, Laboratoire National de Métrologie et d'Essais, 75015 Paris, France*

**Although the general cytotoxicity of selenite is well established, the mechanism by which this compound crosses cellular membranes is still unknown. Here, we show that in *Saccharomyces cerevisiae*, the transport system used opportunistically by selenite depends on the phosphate concentration in the growth medium. Both the high and low affinity phosphate transporters are involved in selenite uptake. When cells are grown at low $P_i$ concentrations, the high affinity phosphate transporter Pho84p is the major contributor to selenite uptake. When phosphate is abundant, selenite is internalized through the low affinity $P_i$ transporters (Pho87p, Pho90p, and Pho91p). Accordingly, inactivation of the high affinity phosphate transporter Pho84p results in increased resistance to selenite and reduced uptake in low $P_i$ medium, whereas deletion of *SPL2*, a negative regulator of low affinity phosphate uptake, results in exacerbated sensitivity to selenite. Measurements of the kinetic parameters for selenite and phosphate uptake demonstrate that there is a competition between phosphate and selenite ions for both $P_i$ transport systems. In addition, our results indicate that Pho84p is very selective for phosphate as compared with selenite, whereas the low affinity transporters discriminate less efficiently between the two ions. The properties of phosphate and selenite transport enable us to propose an explanation to the paradoxical increase of selenite toxicity when phosphate concentration in the growth medium is raised above 1 mM.**

Although toxic at high concentrations, selenium is required in many cells, because it is translationally incorporated as selenocysteine into selenoproteins that perform specific and essential functions (1). Cells must ensure selenium uptake to sustain this metabolism. However, little is known about selenium transport. Because selenium and sulfur are chalcogen elements that have many chemical properties in common, selenium shares metabolic pathways with sulfur. Accordingly, selenate was shown to be taken up by the yeast *Saccharomyces cerevisiae* sulfate permeases (2). Similarly, in plants, selenate is taken up by roots via the high affinity sulfate transporters (3).

On the other hand, specific selenite transporters have never been identified so far. The sulfate ABC transporter of *Escherichia coli* mediates selenite uptake in addition to that of selenate (4). However, repression of the expression of that transporter does not quench selenite uptake completely, indicating that an alternative entry pathway exists for selenite (5). In *S. cerevisiae*, which does not possess selenoproteins, an energy-dependent uptake of selenite, distinct from that of selenate, was reported. Characterization of the kinetics of selenite uptake suggested the existence of two transport systems: a high affinity system at low selenite concentration and a low affinity system at higher concentration (6). Recently, a study of selenite uptake by wheat (*Triticum aestivum*) roots showed it to be an active process competitively inhibited by phosphate, suggesting a role for the plant phosphate transporters in selenite uptake (7). Interestingly, in *S. cerevisiae*, a correlation between resistance to the toxicity of selenite and the expression of a high affinity phosphate transporter has been evidenced (8).

In this study, we asked whether the selenite and phosphate oxyanions, which have similar sizes and charges at pH 6, share the same pathways to enter *S. cerevisiae* cells. Phosphate is an essential nutrient required for numerous biological processes such as biosynthesis of nucleic acids and phospholipids. In *S. cerevisiae*, the inorganic phosphate ($P_i$) acquisition system is composed of five transporters (9). Three of them (Pho87p, Pho90p, and Pho91p) are constitutively transcribed and take up phosphate with low affinity (10). The high affinity transport system, composed of Pho84p and Pho89p, is transcriptionally up-regulated by the phosphate signal transduction (PHO)[2] pathway in response to $P_i$ starvation (11–13). This well characterized regulatory pathway requires the transcription factor Pho4p, whose nuclear or cytoplasmic localization depends on the cyclin/cyclin-dependent kinase complex Pho80p-Pho85p and the cyclin-dependent kinase inhibitor Pho81p. When phosphate is limiting, the cyclin-dependent kinase inhibitor Pho81p inactivates the Pho80p-Pho85p complex, leading to accumulation of unphosphorylated Pho4p in the nucleus and subsequent activation of phosphate-responsive genes (14–17). When phosphate is abundant, Pho4p is phosphorylated by the Pho80p-Pho85p complex and exported to the cytoplasm by the receptor Msn5p, where it becomes unable to activate transcription. One of the genes up-regulated by the PHO pathway is *SPL2*, a negative regulator of the low affinity phosphate transporters (18). Inhibition of the low affinity phosphate transport is likely to occur through a physical interaction with Spl2 because both Pho87p and Pho90p have an SPX (SYG1, pho81, XPR1) domain that has been shown to bind the regulatory protein

[1] To whom correspondence should be addressed. Tel.: 33-1-69-33-49-00; Fax: 33-1-69-33-49-09; E-mail: myriam.lazard@polytechnique.edu.

[2] The abbreviations used are: PHO, phosphate signal transduction; SD, synthetic dextrose; MES, 4-morpholineethanesulfonic acid; ICP/MS, inductively coupled plasma mass spectrometry.

Spl2p (19). Thus, although the low affinity phosphate transporters are not transcriptionally regulated in response to external phosphate availability, their transport activity is inhibited post-transcriptionally following phosphate starvation. Overall, the above regulatory mechanism results in cells that use either the high affinity or the low affinity transport systems, depending on phosphate availability.

In this study, we show that selenite toxicity is dependent on phosphate concentrations in the growth medium. Selenite uptake measurements allow us to establish that both the high affinity phosphate transporter Pho84p as well as the low affinity carriers mediate selenite uptake in *S. cerevisiae* and that phosphate and selenite compete for uptake by both these systems. Primacy of one transport system on the other depends on the phosphate concentration conditions used to grow the cells.

## EXPERIMENTAL PROCEDURES

*Strains and Media*—The *S. cerevisiae* strains used in this study are derived from strain BY4742 (*MAT*a *his3*Δ*1 leu2*Δ *lys2*Δ*0 ura3*Δ*0*). The parental and all the single mutants were purchased from Euroscarf. The double and triple mutant strains were constructed by replacing the entire reading frame of the reference gene with a PCR-generated marker cassette containing either the *URA3kl* or the *LEU2* genes (20). All of the disruptions were verified by PCR analysis. The constructed strains are as follows: Δ*pho87*-Δ*pho90* (BY4742 *pho87*::*KanMX4*; *pho90*::*URA3kl*), Δ*pho87*-Δ*pho91* (BY4742 *pho87*::*KanMX4; pho91*::*URA3kl*), Δ*pho90*-Δ*pho91* (BY4742 *pho90*::*KanMX4*; *pho91*::*URA3kl*), Δ*pho84*-Δ*spl2* (BY4742 *pho84*::*KanMX4*; *spl2*::*URA3kl*), and Δ*pho87*-Δ*pho90*-Δ*pho91* (BY4742 *pho87*::*KanMX4*; *pho90*::*URA3kl*; *pho91*::*LEU2*). Plasmid pRS426 and its derivatives expressing *PHO84* (pPHO84) or *PHO87* (pPHO87) from the *ADH1* promoter are described in Ref. 9. YPD medium contained 1% yeast extract (Difco), 1% Bactotryptone (Difco), and 2% glucose. Standard synthetic dextrose (SD) minimal medium contained 0.67% yeast nitrogen base (Difco), 2% glucose, and 50 mg/liter of histidine, leucine, lysine, and uracil and was buffered at pH 6.0 by the addition of 50 mM MES-NaOH. This medium contained 7.3 mM phosphate. Phosphate-depleted SD medium was prepared as described (21). Then 2% glucose, 50 mg/liter of histidine, leucine, lysine, and uracil, and 50 mM MES were added to the filtered phosphate-depleted medium, and the pH was adjusted to 6.0 with HCl. The medium was filtered through a 0.2-μm filter unit (Nalgene). This medium contained less than 50 μM phosphate (21) and did not support growth of the parental strain. Phosphate was added to this medium at the indicated concentrations from a 100 mM potassium phosphate pH 6.0 solution.

*Toxicity Assays*—The strains were always pregrown overnight at 30 °C in SD medium containing the amount of phosphate used in the following experiments with the exception of strains containing the Δ*pho4* or Δ*pho84* deletions, which do not grow well in low phosphate medium. These strains were pregrown at 0.6 or 0.8 mM phosphate prior to dilution to the desired phosphate concentration and incubated further for at least 4 h.

For growth inhibition, the cells were inoculated in phosphate-defined medium at an $OD_{650}$ of 0.12 and left to grow at 30 °C for 1 h. Then 5 mM $Na_2SeO_3$ (Sigma) was added to half of the cultures, and growth was monitored by following the $OD_{650}$ as a function of time. For viability assays, the cells were inoculated in phosphate-defined medium to obtain an $OD_{650}$ of 0.025 and left to grow at 30 °C. When the $OD_{650}$ reached 0.1, sodium selenite, selenate, or selenide was added to 1 ml of the cultures at the desired final concentration. After 1 h at 30 °C under agitation, the samples were diluted 1000-fold in water. To monitor cell viability, 200-μl aliquots of this dilution were plated in duplicate onto YPD agar plates. The plates were left to grow for 2 days at 30 °C prior to scoring.

*Phosphate Uptake*—The strains were pregrown overnight as indicated above. The cells were inoculated in phosphate-defined medium to obtain an $OD_{650}$ of 0.4 and left to grow at 30 °C. When the $OD_{650}$ reached 1.5, the cells were harvested by centrifugation and washed with SD medium without phosphate. The cells were resuspended in SD medium without phosphate at a density of 1 $OD_{650}$/ml and incubated at 30 °C for 5 min prior to the addition of $[^{32}P]P_i$ (PerkinElmer Life Science). Uptake measurements were initiated by the addition of 0.1 ml of $[^{32}P]P_i$ (final concentrations from 5 to 7500 μM; specific activity between $10^3$ and $10^6$ dpm·nmol$^{-1}$) to 0.9 ml of cell suspension. The assays were terminated at 3 and 6 min by the addition of 1 ml of ice-cold 0.5 M phosphate, pH 6.0. Cell suspensions were then filtered using 0.45-μm nitrocellulose filters (Schleicher & Schuell) and washed twice with 3 ml of 0.25 M phosphate, pH 6.0. The radioactivity retained on the filter was measured by liquid scintillation counting. The rates of transport are given in pmol/min and per $OD_{650}$. The $K_m$ and $V_{max}$ values were derived from iterative nonlinear fits of the theoretical Michaelis equation to the experimental values, using the Levenberg-Marquardt algorithm as described previously (22). When experimental values could not be fitted to a single hyperbola, a partition method was employed, as recommended (23). $K_{m\,1}$ and $V_{max\,1}$ values were derived from the experimental data obtained for phosphate concentrations ranging from 5 to 50 μM. The theoretical contribution of the first uptake system was subtracted from the subsequent experimental values (100–5000 μM). The resulting data were used to determine $K_m$ and $V_{max}$ values for the second uptake system.

*Selenite Uptake*—The strains were pregrown overnight as indicated above. The cells were inoculated in phosphate-defined medium to obtain an $OD_{650}$ of 0.4 and left to grow at 30 °C. When the $OD_{650}$ reached 1.5, the cells were harvested by centrifugation and washed with SD medium without phosphate. The cells were resuspended in SD medium without phosphate at a density of 5 $OD_{650}$/ml and incubated at 30 °C for 10 min prior to the addition of $Na_2SeO_3$. For determination of the kinetic parameters for selenite uptake, selenite concentrations were in the range 1–20 mM. At time intervals, 10 ml of cell suspension were removed from the incubation mixture, and the reaction was stopped by the addition of 1 ml of ice-cold 1 M phosphate, pH 6.0. The samples were centrifuged (5000 × *g* for 5 min), washed twice with 10 ml of water, and lyophilized.

Selenium content of the cells was determined by inductively coupled plasma mass spectrometry (ICP/MS) on a Thermo Fisher PQ Excell quadripole spectrometer in the collision cell mode. The mass of each yeast sample was estimated by

the difference of weight between the filled and empty microtubes (microbalance Sartorius ME 36S, range 30 g/1 $\mu$g). The yeast samples were digested with 3 ml of $HNO_3$ (67–69%; Plasmapur SGS) and 1 ml of $H_2O_2$ (30%; Suprapur Merck) in a closed vessel microwave oven (Ethos 900; Thermo Fisher). The residues were then diluted with 10 ml of pure water. The ICP/MS mass spectrometer was calibrated (range, 0–200 $\mu$g/liter) against a reagent blank solution, and several selenium standard solutions were obtained after dilutions from a concentrated certified standard (selenium, 999 ± 5 mg/liter; Certipur Merck). The data were recorded for the four selenium isotopes $^{76}$Se, $^{78}$Se, $^{80}$Se, and $^{82}$Se. Selenium concentrations calculated from each isotope were analogous. The results are the means of data obtained on each isotope and are expressed in $\mu$g of selenium·g$^{-1}$ (dry weight). Conversion in pmol·$OD_{650}^{-1}$ was made using a mean atomic mass of 79 and by considering that 1 g of yeast (dry weight) corresponds to 6200 $OD_{650}$.

## RESULTS

*Effect of Phosphate on Selenite Toxicity*—The toxicity of selenite in yeast is well documented, although the mechanisms of toxicity are less well understood (24, 25). Selenite uptake is the first step in selenium metabolism that ultimately leads to toxicity. To investigate the possibility that selenite enters yeast cells via the orthophosphate transport system, the influence of the concentration of phosphate in the growth medium on the toxicity of sodium selenite was analyzed. We compared the effect of the addition of 5 mM of sodium selenite on the growth of yeast cells (strain BY4742) cultivated in the presence of increasing concentrations of potassium phosphate (SD with 0.1, 0.2, 0.4, or 0.8 mM $P_i$ or standard high $P_i$ SD (7.3 mM $P_i$)) (Fig. 1*A*). In the absence of selenite, whatever the concentration of phosphate, growth rates were identical ($t_{½}$ = 135 min at 30 °C). We observed, however, that in the medium supplemented with 0.1 mM phosphate, upon reaching the late exponential phase, phosphate depletion became limiting for cell growth. In the presence of 5 mM selenite, growth rates and plateau values were clearly dependent on phosphate concentrations. Up to 0.8 mM $P_i$, the cells were much more affected by selenite toxicity in the medium containing the lowest phosphate concentration. Surprisingly, at high phosphate concentration, selenite toxicity increased.

To determine whether this inhibition of growth was due to increased selenite lethality, cell viability was assayed after a short term exposure to selenite. The cells, grown exponentially in medium containing various concentrations of phosphate, were incubated for 1 h in the same medium with 5 or 10 mM selenite, diluted, and plated on rich medium plates (high phosphate). The survival rates were determined by counting colonies after 2 days growth at 30 °C (Fig. 1*B*). In a medium containing 0.1 mM $P_i$, exposure to 5 mM selenite reduced cell survival by nearly 90%. In contrast, at 0.4 mM $P_i$, more than 80% of the cells were resistant to 5 mM selenite. When the phosphate concentration was raised further, viability of the cells decreased to reach ~50% in high phosphate medium. These results show that selenite not only inhibits the growth of yeast cells but also induces mortality.

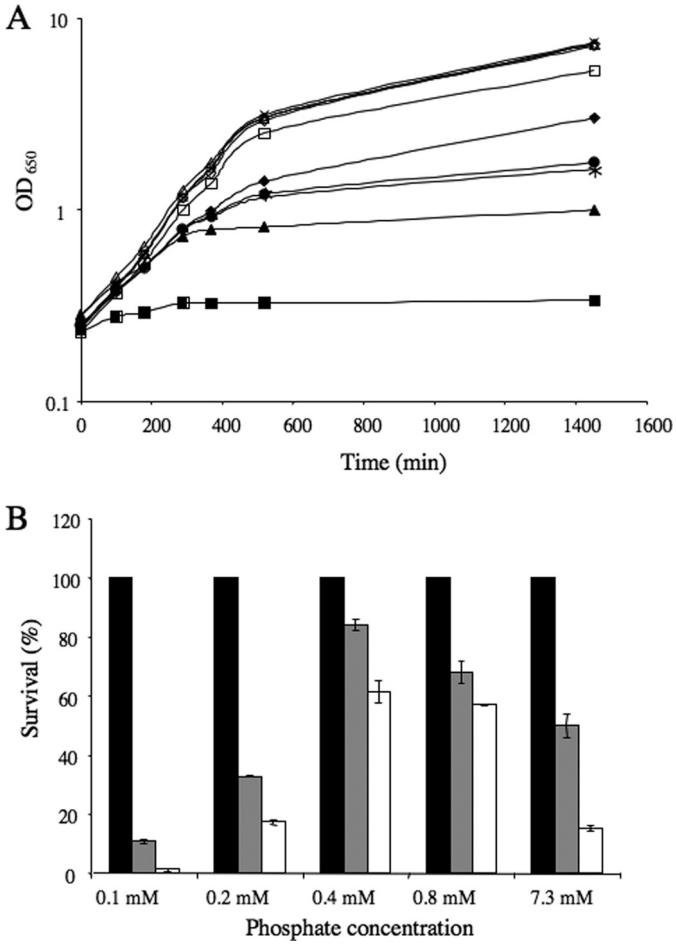


FIGURE 1. **Selenite toxicity is dependent on phosphate concentration.** *A*, cells (strain BY4742) were grown at 30 °C in phosphate-depleted SD medium supplemented with phosphate as follows: 0.1 mM (□, ■), 0.2 mM (△, ▲), 0.4 mM (○, ●), 0.8 mM (◆, ◇), and 7.3 mM (×, *). The cultures were divided in two, and 5 mM $Na_2SeO_3$ was added to one sample (*filled symbols*). Cell growth was monitored by measuring the $OD_{650}$ at various times. *B*, cells (strain BY4742) were incubated in SD medium supplemented with the indicated phosphate concentration. When the $OD_{650}$ reached 0.1, $Na_2SeO_3$ was added to the cultures (0 mM, *black bars*; 5 mM, *gray bars*; 10 mM, *white bars*). After 1 h of incubation at 30 °C, the samples were diluted and plated onto YPD-agar. Cell viability was determined after 2 days of growth at 30 °C. The results are expressed as percentages of survival compared with control samples incubated in the absence of selenite. The *error bars* represent the means and ranges of two independent experiments.

In a previous paper, we showed that extracellular reduction of selenite into hydrogen selenide ($HSe^-$) led to increased cellular accumulation and toxicity of selenium (26). To determine whether sodium selenide toxicity was dependent on phosphate concentration, we measured cell survival after a 5-min exposure to 40 $\mu$M $Na_2Se$ in medium containing increasing concentrations of $P_i$. This resulted in ~50% mortality of the cells, independently of phosphate concentration, suggesting that these two forms of inorganic selenium (selenite and selenide) are internalized by different pathways, as suggested previously (27). Because our sample of sodium selenite might contain traces of selenate, sodium selenate toxicity was also assayed. Exposure to 5 mM selenate resulted in low mortality (<20%), irrespective of phosphate concentration. Thus, the toxicity of selenite cannot originate from trace amounts of selenate contaminating the selenite solution.

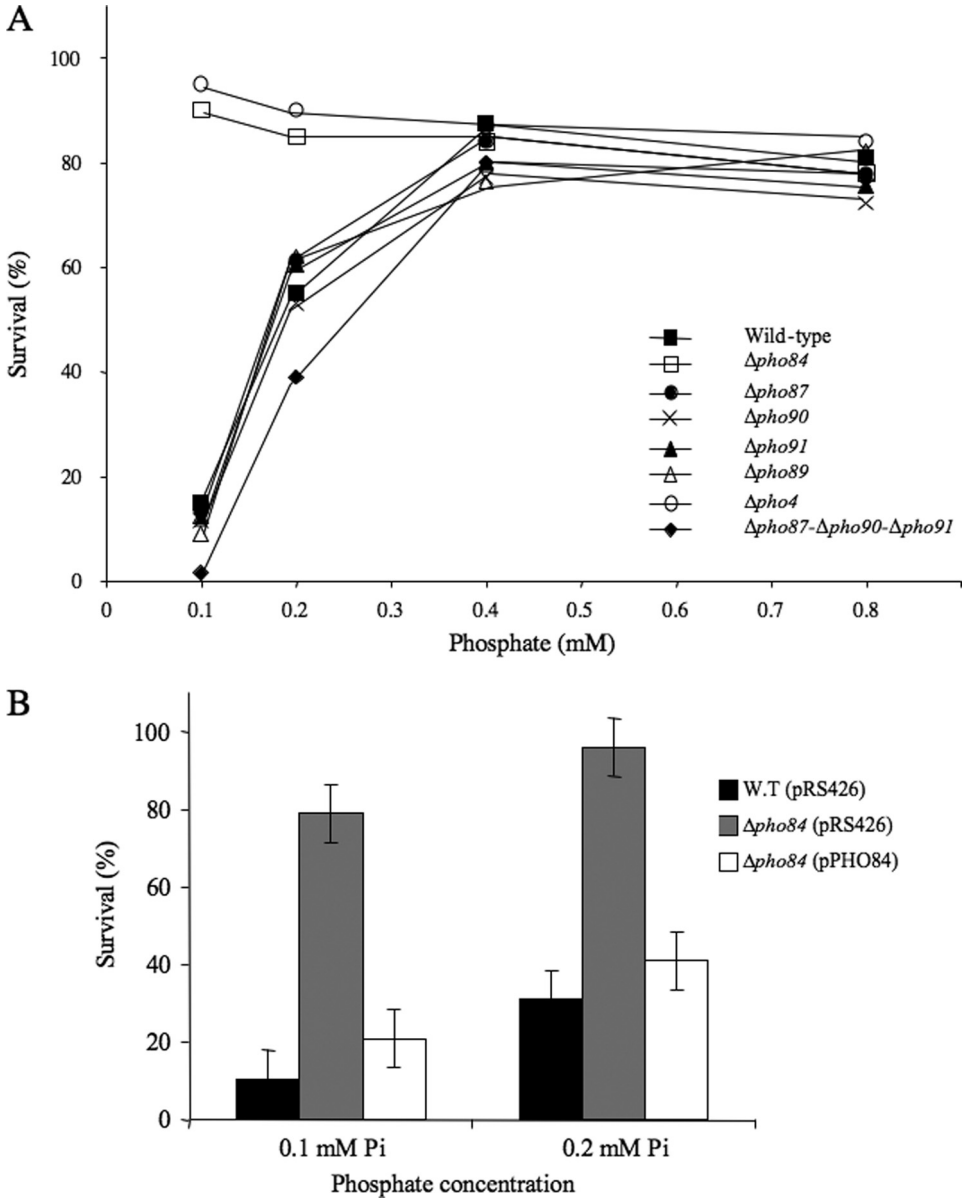


FIGURE 2. **Selenite toxicity in mutants of the phosphate transport pathway.** *A*, various strains, as indicated in the figure, were grown at 30 °C in SD medium supplemented with the indicated phosphate concentration. When the $OD_{650}$ reached 0.1, 5 mM $Na_2SeO_3$ was added to the cultures. After 1 h of incubation at 30 °C, the samples were diluted and plated onto YPD-agar. Cell viability was determined after 2 days growth at 30 °C. The results are expressed as percentages of survival compared with control samples incubated in the absence of selenite. The values are the means of at least three independent experiments. Standard deviations between these experiments were lower than 15%. *B*, cells expressing either the control plasmid pRS426 or pPHO84 expressing *PHO84* from the *ADH1* promoter were grown and treated as in *A*. The *error bars* represent the means and ranges of two independent experiments.

*Selenite Toxicity in Mutants of the PHO Pathway*—In a first set of experiments, the toxicity of selenite was analyzed in $P_i$-limited medium. Viability assays were performed in strains disrupted for one of the phosphate transporter genes (*PHO84*, *PHO87*, *PHO89*, *PHO90*, and *PHO91*). The cells were pregrown with increasing $P_i$ concentrations, exposed to 5 mM selenite, plated, and scored to assess cell survival (Fig. 2*A*). Whatever the concentration of phosphate in the range 0.1–0.8 mM, each of the single mutants in the low affinity carriers (Δ*pho87*, Δ*pho90*, and Δ*pho91*) displayed curves similar to that of the parental strain, with high selenite toxicity at low $P_i$ concentrations. However, a triple mutant Δ*pho87*-Δ*pho90*-Δ*pho91* was slightly more sensitive to selenite than the wild-type strain. At 0.1 mM $P_i$, survival was 1.4% for the triple mutant as compared with 15% for wild type. In contrast, the Δ*pho84* strain was very resistant to selenite toxicity at low $P_i$ concentrations. The Δ*pho4* strain, in which *PHO84* is not expressed, was also very resistant to selenite toxicity at low $P_i$ concentrations. As shown in Fig. 2*B*, expression, in the Δ*pho84* strain, of *PHO84* from the constitutive *ADH1* promoter restored selenite toxicity values close to those observed in the wild type. These results suggest that Pho84p plays a major role in the toxicity of selenite, at least up to 0.4–0.5 mM phosphate.

In the case of a Δ*pho89* strain, the survival curve was similar to that of the wild type. This indicates that, under our experimental conditions, this transporter is not involved in selenite toxicity. However, both the transcription and the activity of Pho89p are strongly pH-dependent, with an optimum pH of >8.0 (13). Although no effect of the inactivation of its gene was observed, a potential role for Pho89p in selenite toxicity cannot be excluded but must be studied in different conditions. For this reason, this high affinity transporter was not considered in the remainder of our study.

*Selenite Uptake Is Reduced in a* Δ*pho84 Mutant*—The uptake of selenite by BY4742 yeast cells was measured using cells grown in $P_i$-limited conditions (0.3 mM). The cells were washed and resuspended in SD medium without $P_i$. The cell suspensions were incubated at 30 °C in the presence of various concentrations of selenite (1–10 mM), and aliquots were removed at various times to measure the amount of total incorporated selenium (Fig. 3*A*). Cellular accumulation of selenium was linear over a period of at least 10 min. The rate of selenite uptake, as a function of selenite concentration, could be adequately described by the Michaelis-Menten equation, allowing us to derive a $K_m$ of 4 ± 1 mM and a $V_{max}$ of 67 ± 4 μg of selenium·$g^{-1}$·$min^{-1}$ (or 136 pmol of selenium·$OD^{-1}$·$min^{-1}$) (Fig. 3*B*). The rate of selenite uptake, at the selenite concentration of 5 mM, was 76 pmol of selenium·$OD^{-1}$·$min^{-1}$ (Table 1). The uptake of 5 mM selenite, by the Δ*pho84* mutant assayed in the same conditions, was only 11 pmol of selenium·$OD^{-1}$·$min^{-1}$, indicating that deletion of *PHO84* drastically

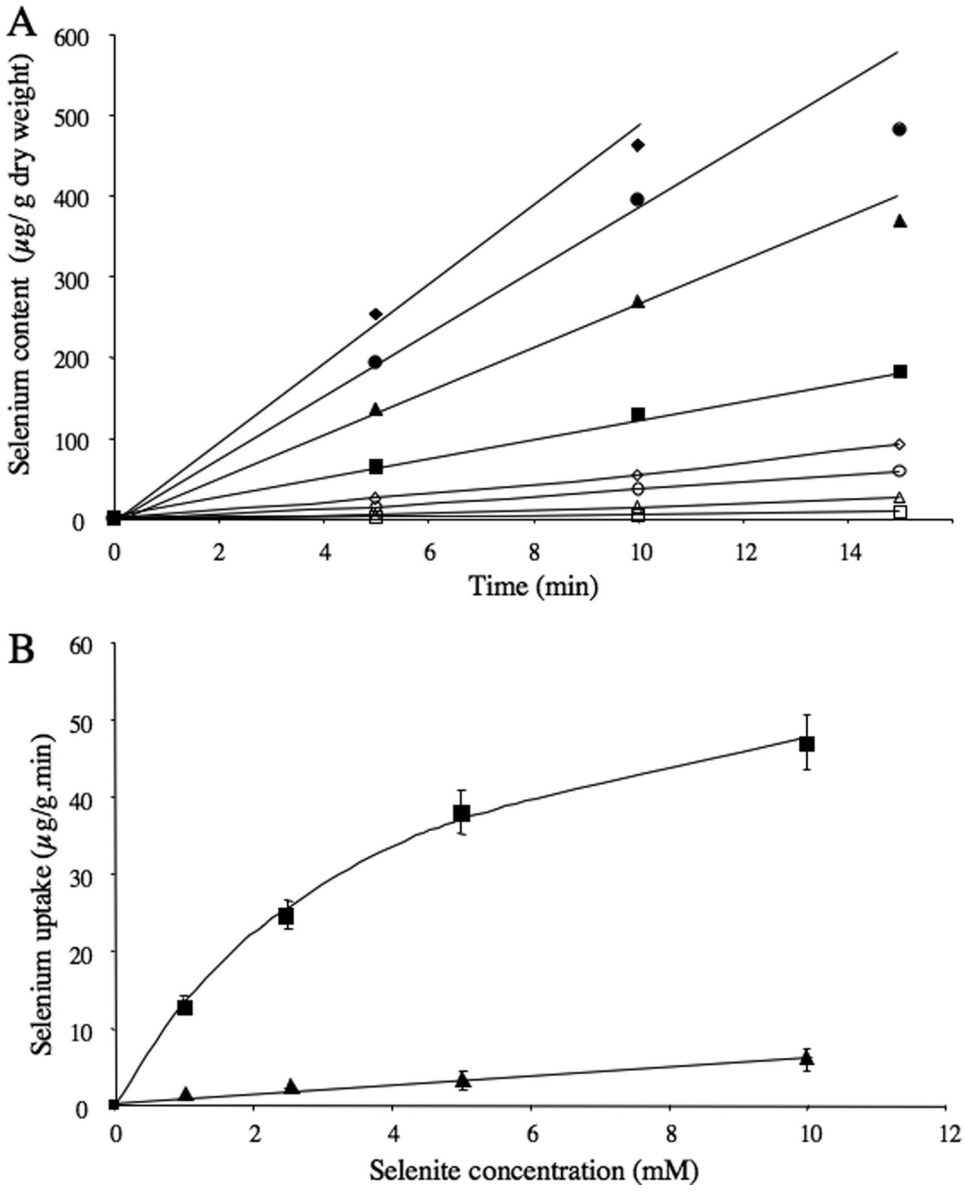


FIGURE 3. **Uptake of selenium by BY4742 cells grown in SD medium supplemented with 0.3 mM $P_i$.** *A*, cells were harvested by centrifugation, washed, and resuspended in SD medium without phosphate. Uptake of 1 mM (□, ■), 2.5 mM (▲, △), 5 mM (○, ●), and 10 mM (◆, ◇) $Na_2SeO_3$ was measured as described under "Experimental Procedures," in the absence (*filled symbols*) or the presence (*open symbols*) of 0.5 mM potassium phosphate. The samples were analyzed for their total selenium content. *B*, rates of selenite uptake in the absence of phosphate (■), determined from *A*, were fitted to the Michaelis-Menten equation as described under "Experimental Procedures." In the presence of 0.5 mM phosphate (▲), the rate of selenite uptake increased roughly linearly with the selenite concentration.

reduced selenite uptake. These results show that, in $P_i$-limited conditions, transport of selenite is ensured mostly by Pho84p. In addition, the kinetics of selenite uptake were measured in the presence of 0.5 mM $P_i$ in the uptake medium (Fig. 3*B* and Table 1). Whatever the selenite concentration, accumulation of selenium was reduced at least 10 times, showing that selenite uptake by Pho84p is inhibited by phosphate.

*Inhibition of Phosphate Uptake by Selenite in Cells Grown in Low $P_i$ Conditions*—Because the addition of phosphate in the assay reduced selenium accumulation, phosphate uptake was expected to be inhibited by selenite. Therefore, [$^{32}$P]$P_i$ transport kinetic parameters were measured in the presence of increasing concentrations of selenite (Table 2).

First, we analyzed phosphate uptake in conditions identical to those previously used to measure selenite uptake (strain BY4742, 0.3 mM $P_i$). The rate of phosphate accumulation at concentrations ranging from 5 to 500 $\mu$M could be fitted to a single Michaelis-Menten equation, giving a $V_{max}$ of 860 pmol of $P_i$ incorporated per min/$OD_{650}$ and a $K_m$ of 20 $\mu$M. The $K_m$ for $P_i$ uptake is in the micromolar range, suggesting that only high affinity phosphate transport is operative at this phosphate concentration. To support this idea, we compared the rate of phosphate uptake in the wild-type strain and the Δ*pho84* mutant grown in 0.3 mM $P_i$, using a phosphate concentration of 0.3 mM in the assay. Rates of 840 and 43 pmol of $P_i \cdot OD^{-1} \cdot min^{-1}$ were determined, respectively. These results indicate that in the growth conditions used above, Pho84p is the major contributor to phosphate uptake. Kinetic studies were also performed in the presence of 1, 2.5, 5, and 10 mM selenite. The results show that selenite competitively inhibits phosphate uptake by BY4742 cells with a $K_i$ of 4.6 mM (Table 2). It is noteworthy that this value is close to the $K_m$ determined for selenite uptake. This is in agreement with the conclusion that the same transporter, Pho84p, is responsible for the major part of the uptake of both phosphate and selenite. In a previous paper (8), the authors found that selenite did not compete with phosphate transport. However, they did not go beyond a 10-fold molar excess of selenite, which may not have been enough to observe an effect, because of the large difference of $K_m$ for phosphate or selenite transport.

We also measured the $K_i$ of selenite toward phosphate uptake in the Δ*pho87*-Δ*pho90*-Δ*pho91* strain. Experimental conditions were identical to those used above with the wild-type strain. As expected, the $K_m$ for phosphate (25 $\mu$M) and the $K_i$ for selenite (5.5 mM) were similar to those determined with the BY4742 strain, which confirms that the selenite transport kinetic values determined above correspond to Pho84p-mediated uptake.

*Selenite Toxicity in High Phosphate Medium*—Transport of selenite by Pho84p cannot explain the increase in selenite toxicity observed with cells grown in high $P_i$ conditions, because Pho84p is not significantly expressed in these conditions (11,

18). In agreement with this idea, when assayed for cell survival after exposure to 5 or 10 mM selenite, the single mutants in the high affinity transporter genes (*PHO84* and *PHO89*), as well as the Δ*pho4* and Δ*spl2* strains, grown in high phosphate medium, displayed selenite survival values comparable with those of the wild-type strain (Fig. 4). In contrast, single mutant strains in the low affinity $P_i$ transporters, as well as the double and triple mutants, were more resistant to selenite. An increased resistance of the Δ*pho87*, Δ*pho90*, and Δ*pho91* mutants has been previously reported by Pinson *et al.* (8). Several studies have shown that mutations in the low affinity phosphate transporters resulted in derepression of the PHO-regulated genes (8, 10, 28), leading to increased transcription of *PHO84* and to inactivation of the low affinity transporters through derepression of *SPL2*. Thus, in these mutants, Pho84p is responsible for most of the phosphate uptake, whatever the phosphate conditions.

To confirm the induction of the PHO pathway upon the deletion of a single low affinity transporter gene, we compared kinetic parameters for phosphate uptake in wild-type cells grown in high $P_i$ medium and in a Δ*pho87* strain (Table 2). Values corresponding to low affinity $P_i$ transport were observed for the wild-type strain ($K_m$ = 4.3 mM, $V_{max}$ = 1600 pmol of $P_i \cdot OD^{-1} \cdot min^{-1}$). This $K_m$ was higher than that usually associated with the low affinity $P_i$ transporters (4 mM instead of 0.2–0.8 mM). This difference might be related to the pH conditions of our assay, because $P_i$ transport is usually assayed at pH 4.5 instead of pH 6.0. As expected, deletion of *PHO87* led to a $K_m$ in the micromolar range showing that, in this mutant, only high affinity phosphate transporters are functional. Another circumstance in which the PHO regulon is constitutively expressed is in the Δ*pho85* mutant. In such a strain, inactivation of the Pho85p kinase leads to accumulation of unphosphorylated Pho4p in the nucleus and thus to constitutive activation of the PHO pathway. We found that this strain was also very resistant to selenite toxicity at high $P_i$ concentrations (Fig. 4). Finally, overexpression of plasmid-encoded *PHO84*, in the Δ*pho87* mutant, did not alter selenite survival values, whereas expression of *PHO87* reduced them to wild-type levels.

Altogether, these results suggest that, when phosphate is abundant in the medium, strains expressing the low affinity transporters (wild type, Δ*pho4*, Δ*pho89*, Δ*pho84*, Δ*spl2*, and Δ*pho87* (pPHO87)) are more sensitive to selenite toxicity than strains using mostly Pho84p to uptake phosphate (Δ*pho85*, Δ*pho87*, Δ*pho90*, Δ*pho91*, and Δ*pho87* (pPHO84)). One possible explanation is that suppression of low affinity transport influences intracellular selenite metabolism, leading to lower $H_2Se$ production and, therefore, lower toxicity. Because down- or up-regulation of the sulfate assimilation pathway might interfere with selenium metabolism, we asked whether this pathway was altered in the deletion mutants. For this purpose, the strains were grown on Pb(II)/sulfate plates (29). Wild-type and single deletion mutants in the low affinity $P_i$ transport system displayed equivalent coloring (result not shown), suggesting that these mutations do not interfere with the metabolism of sulfate.

A more likely hypothesis is that low affinity $P_i$ transporters are able to uptake selenite and that, in high $P_i$ medium, they are more efficient than Pho84p in importing this ion. The following experiments were performed to assess this idea.

*Selenite Toxicity in a Δspl2 Strain*—To establish that the low affinity transporters are involved in the uptake of selenite, we used a Δ*spl2* strain grown in $P_i$-limited medium. In this strain, low affinity transport is active whatever the phosphate concentration. First, we determined the contribution of each transport system to total phosphate uptake in the Δ*spl2*

**TABLE 1**

**Rates of selenite uptake and inhibition by phosphate of wild-type and $P_i$ transporter-defective strains in the presence of 5 mM selenite**

The selenite uptake measurements were performed as described under "Experimental Procedures." The errors are standard deviations. ND, not determined.

| $P_i$ concentration in the growth medium | Strain | $V$ | |
|---|---|---|---|
| | | 0 mM $P_i$[a] | 0.5 mM $P_i$[a] |
| | | pmol of $SeO_3^{2-} \cdot min^{-1} \cdot OD_{650}^{-1}$ | |
| 0.3 mM | BY4742 | 76 ± 10 | 6.4 ± 1 |
| 7.3 mM | BY4742 | 27 ± 4 | 24 ± 4 |
| 0.3 mM | Δ*pho84* | 11 ± 4 | ND |
| 7.3 mM | Δ*pho84* | 20 ± 4 | ND |
| 0.3 mM | Δ*pho84*-Δ*spl2* | 21 ± 4 | ND |
| 7.3 mM | Δ*pho84*-Δ*spl2* | 21 ± 3 | ND |
| 7.3 mM | Δ*pho87* | 31 ± 5 | 14 ± 4 |
| 7.3 mM | Δ*pho87* (pPHO87) | 30 ± 4 | 26 ± 3 |
| 7.3 mM | Δ*pho85* | 87 ± 10 | 11 ± 2 |

[a] Concentration of phosphate in the uptake medium.

**TABLE 2**

**Phosphate uptake parameters and selenite inhibition constants of wild-type and $P_i$ transporter-defective strains**

The uptake experiments were performed as described under "Experimental Procedures" on two independently grown cultures, apart from BY4742 grown in 0.3 mM $P_i$ (four independent experiments), BY4742 grown in 7.3 mM $P_i$, and Δ*spl2* grown in 0.3 mM $P_i$ (three independent experiments). Seven different concentrations of phosphate were used to determine the kinetic parameters. The $P_i$ concentrations were in the range 5–500 μM for high affinity uptake and 0.1–7.5 mM for low affinity uptake measurements. To determine the phosphate uptake parameters in the Δ*spl2* strain, 10 concentrations of phosphate were used (5–5000 μM). Selenite inhibition constants were determined from the measurements of the apparent $K_m$ for phosphate in the presence of 2.5, 5, and 10 mM selenite. The $K_i$ was deduced from the slope of the plot of $K_m^{app}$ as a function of selenite concentration. The errors are standard deviations.

| $P_i$ concentration | Strain | $V_{max}$ | $K_m$ | $K_i$ |
|---|---|---|---|---|
| | | pmol $P_i \cdot min^{-1} \cdot OD_{650}^{-1}$ | μM | mM |
| 0.3 mM | BY4742 | 860 ± 80 | 20 ± 4 | 4.6 ± 1.7 |
| 0.3 mM | Δ*pho87*-Δ*pho90*-Δ*pho91* | 2000 ± 300 | 25 ± 3 | 5.5 ± 1.8 |
| 0.3 mM | Δ*spl2* | $V_{max\,1}$ 600 ± 140 | $K_{m\,1}$ 19 ± 6 | |
| | | $V_{max\,2}$ 1700 ± 400 | $K_{m\,2}$ 4000 ± 1300 | |
| 0.3 mM | Δ*pho84* | 700 ± 150 | 5000 ± 1600 | |
| 0.3 mM | Δ*pho84*-Δ*spl2* | 1400 ± 300 | 5900 ± 1800 | |
| 7.3 mM | BY4742 | 1600 ± 400 | 4300 ± 1600 | 9.0 ± 2.0 |
| 7.3 mM | Δ*pho87* | 440 ± 50 | 25 ± 6 | |
| 7.3 mM | Δ*pho84* | 1700 ± 200 | 6000 ± 2000 | 9.5 ± 2.0 |
| 7.3 mM | Δ*pho84*-Δ*spl2* | 2000 ± 500 | 5800 ± 2000 | |

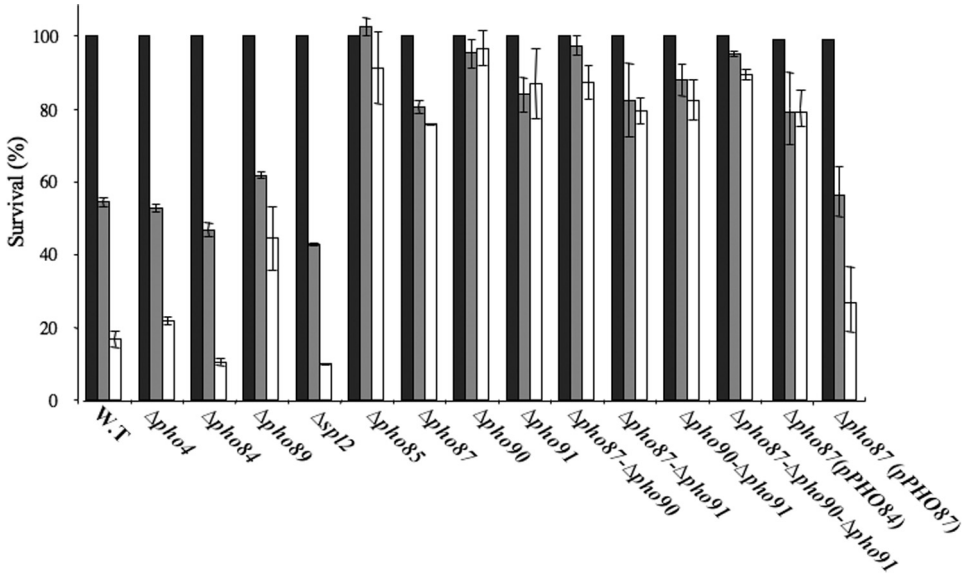


FIGURE 4. **Selenite toxicity in high $P_i$ medium.** The cells were incubated in SD medium. When the $OD_{650}$ reached 0.1, $Na_2SeO_3$ was added to the cultures (0 mM, *black bars*; 5 mM, *gray bars*; 10 mM, *white bars*). After 1 h of incubation at 30 °C, the samples were diluted and plated onto YPD-agar. Cell viability was determined after 2 days growth at 30 °C. The results are expressed as percentages of survival compared with control samples incubated in the absence of selenite. The *error bars* represent the means and ranges of two independent experiments. *W.T*, wild type.

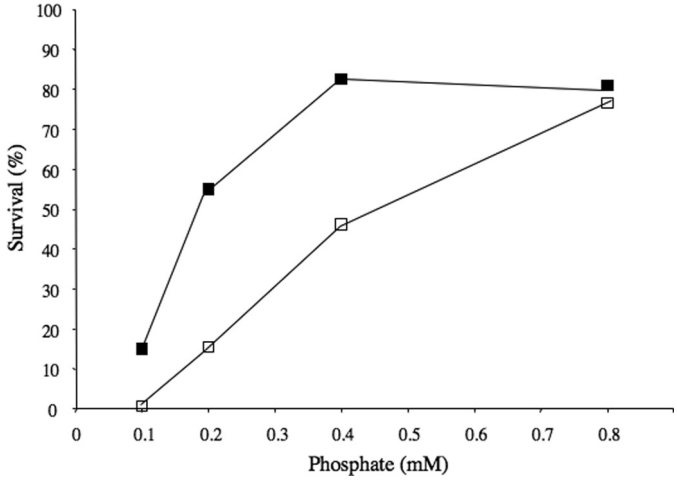


FIGURE 5. **Sensitivity to selenite toxicity of a Δ*spl2* strain grown in low $P_i$ medium.** Strains BY4742 (■) and Δ*spl2* (□) were grown at 30 °C in SD medium supplemented with the indicated phosphate concentrations. When the $OD_{650}$ reached 0.1, 5 mM $Na_2SeO_3$ was added to the cultures. After 1 h of incubation at 30 °C, the samples were diluted and plated onto YPD-agar. Cell viability was determined after 2 days of growth at 30 °C. The results are expressed as percentages of survival compared with control samples incubated in the absence of selenite. The values are the means of at least three independent experiments. Standard deviations between these experiments were lower than 15%.

mutant. As expected from previous studies (18), deletion of *SPL2* resulted in activation of the low affinity transporters (Table 2). The observed values of $[^{32}P]P_i$ incorporated could not be fitted with a single hyperbola but were correctly fitted with a double hyperbola curve. The results show that the high affinity system ($K_{m\,1}$ = 19 μM) uptakes phosphate with a $V_{max\,1}$ of 600 pmol of $P_i \cdot OD^{-1} \cdot min^{-1}$. Activation of the low affinity transporters allowed us to determine a $K_{m\,2}$ of 4 mM and a $V_{max\,2}$ of 1700 pmol of $P_i \cdot OD^{-1} \cdot min^{-1}$ for low affinity $P_i$ transport.

Then we assayed selenite toxicity in the Δ*spl2* strain. As shown in Fig. 5, inactivation of *SPL2* resulted in increased selenite sensitivity, as compared with the wild-type cells. At 0.1 mM $P_i$, survival of the Δ*spl2* cells exposed to 5 mM selenite was less than 1%. These results show that deletion of *SPL2* leads to the activation of the low affinity transport system with a concomitant increase in selenite toxicity.

*Selenite Uptake by the Low Affinity Transport System*—To demonstrate that the low affinity phosphate transporters can import selenite, we compared the initial rates of selenite uptake at a fixed selenite concentration of 5 mM in Δ*pho84* and Δ*pho84*-Δ*spl2* cells grown in low $P_i$ medium (Table 1). Phosphate uptake was also determined in the same conditions (Table 2). In the strain that expresses Spl2p, activation of the PHO pathway led to down-regulation of the low affinity transporters. As a consequence, the $V_{max}$ of $P_i$ uptake was twice higher in the Δ*pho84*-Δ*spl2* strain than in the Δ*pho84* strain. This higher activity of the low affinity transporters was paralleled by a 2-fold increase of selenite uptake, establishing that the low affinity transporters are able to carry selenite inside cells.

Selenite uptake by the low affinity transporters was also measured in cells grown in high $P_i$ medium. In these conditions, the wild-type, Δ*pho84*, and Δ*pho84*-Δ*spl2* strains exhibited phosphate uptake rate values very similar to that measured with the Δ*pho84*-Δ*spl2* strain grown in low $P_i$ medium (Table 2). The rates of selenite uptake by the three strains grown in high $P_i$ medium were also very similar to that measured with the Δ*pho84*-Δ*spl2* strain grown in low $P_i$ medium (Table 1).

Finally, we determined the kinetic parameters for selenite uptake in the wild-type strain grown in high $P_i$ conditions. A $K_m$ of 7.7 ± 3 mM and a $V_{max}$ of 40 ± 8 μg of selenium·$g^{-1}$·$min^{-1}$ (or 81 pmol of selenium·$OD^{-1}$·$min^{-1}$) were determined. Measurement of phosphate uptake in the presence of various concentrations of phosphate and selenite indicated that selenite competitively inhibited phosphate uptake, with a $K_i$ value close to 9 mM (Table 2). This $K_i$ value is comparable with the $K_m$ for selenite transport by low affinity carriers, as measured in selenite uptake experiments. This result confirms that the low affinity $P_i$ transport system is competent for selenite transport.

*Phosphate Inhibition of Selenite Uptake*—The experimental kinetic values determined above allow us to calculate a ratio between the $K_m$ values for selenite and phosphate of ~250 for Pho84p and ~2 for the low affinity transporters. Therefore, we anticipated that, in high $P_i$ medium, phosphate would inhibit selenite uptake much more efficiently in strains expressing Pho84p than in strains expressing the low affinity system. To verify this prediction, we compared the rates of sel-

**TABLE 3**

**Efficiency and selectivity of phosphate transporters for phosphate and selenite uptakes**

The values are deduced from the data in Table II for $P_i$ uptake and from values given in the text for selenite uptake.

| $P_i$ transporter expressed | Strain and growth conditions | $V_{max}/K_m$ for $P_i$ | $V_{max}/K_m$ for selenite | Selectivity[a] |
|---|---|---|---|---|
| | | *pmol OD$^{-1}$·min$^{-1}$·mM$^{-1}$* | *pmol OD$^{-1}$·min$^{-1}$·mM$^{-1}$* | |
| Pho84p | BY4742 and 0.3 mM $P_i$ | 43000 | 34 | 1260 |
| Low affinity | BY4742 and 7.3 mM $P_i$ | 370 | 10.5 | 35 |

[a] Selectivity is defined as the ratio between the $V_{max}/K_m$ for $P_i$ uptake and that for selenium uptake.

enite uptake in the presence of 0.5 mM $P_i$ in the assay of Δ*pho87* and Δ*pho85* cells to those of strains expressing low affinity transporters (wild type and Δ*pho87* (pPHO87)). The data reported in Table 1 show that, indeed, selenite uptake was severely inhibited by phosphate in Pho84p-expressing strains, whereas little inhibition was observed in strains expressing low affinity $P_i$ transporters. These results indicate that, when extracellular $P_i$ is abundant, selenite is taken up more efficiently by the latter transporters than by Pho84p.

## DISCUSSION

Active selenite transport by *S. cerevisiae* has already been reported (6), but no transporter had been identified so far. In this study, we demonstrate that the high affinity phosphate transporter Pho84p, as well as the low affinity carriers, are able to uptake sodium selenite in *S. cerevisiae*. The transport system used opportunistically by selenite depends on the phosphate concentration in the medium. At low $P_i$ concentrations (up to 0.4 mM), Pho84p is the major contributor to selenite uptake. When phosphate is abundant, the role of Pho84p becomes negligible, and selenite is internalized through one or all of the low affinity $P_i$ transporters. Other toxic metalloids are taken up adventitiously by the existing transport system. For instance, the phosphate transport system is also known to take up arsenate in both prokaryotes and eukaryotes (30, 31). Another example is that of the sulfate transporters of yeast and plants that have low selectivity for sulfate *versus* analogous selenate or chromate (2, 3, 32).

Selenite uptake measurements in cells expressing either the high affinity $P_i$ transporter Pho84p or the low affinity Pho87p, Pho90p, and Pho91p indicate that both systems are able to transport selenite inside the cells. Comparison of the $V_{max}/K_m$ of the transporters for $P_i$ or for selenium (Table 3) shows that Pho84p is slightly more efficient than the low affinity carriers for selenite transport. However, Pho84p has a much higher affinity for $P_i$ than for selenium. Thus, the high affinity transporter is very selective for $P_i$, whereas the low affinity system is much less discriminating.

Selenite toxicity results correlate well with selenium uptake measurements, indicating that mortality of *S. cerevisiae* cells is directly dependent on the amount of internalized selenium by the phosphate transport system. In the wild-type strain grown in very low $P_i$ conditions (0.1 mM), selenite toxicity is high. When the phosphate concentration in the medium is increased up to 0.4 mM $P_i$, selenite toxicity is reduced. This effect is easily accounted for by phosphate inhibition of the Pho84p-mediated selenite uptake. When phosphate concentration in the culture medium is further increased, the transport of phosphate (and of selenite) is progressively taken over by the low affinity carriers. Because these carriers are less specific, the advantage of phosphate over selenite (in term of $V_{max}/K_m$) is reduced, and selenite uptake/toxicity increases. This mechanism implies that, at very high phosphate concentrations, selenite resistance should improve again. This effect was, indeed, observed previously (8). In the Δ*pho84* strain grown in low $P_i$ medium, resistance to selenite toxicity can be attributed to the concomitant inactivation of *PHO84* and down-regulation of low affinity transport that affects both phosphate and selenite transport (Tables 1 and 2) and thus results in low selenite (and phosphate) uptake.

The triple mutant Δ*pho87*-Δ*pho90*-Δ*pho91* was more sensitive to selenite than the wild-type strain in $P_i$-limited medium. In agreement with previous studies (10), we observed that in $P_i$-limited medium the triple-disruptant strain exhibited a 2-fold higher $V_{max}$ of $P_i$ uptake than the wild-type strain (Table 2). It was shown that the higher activity in this strain was accompanied by enhanced transcription of the *PHO84* gene (10). Increased activity of Pho84p, in phosphate and selenite transport, explains the higher sensitivity of this strain to selenite as compared with the wild-type cells. Another strain that is very sensitive to selenite at low $P_i$ concentrations is the Δ*spl2* strain. In this case, loss of the regulation by Spl2p results in activation of low affinity transporters. In this strain, both the high and low affinity $P_i$ transport systems are active in $P_i$-limited conditions, resulting in higher selenite uptake and toxicity.

On the contrary, in high $P_i$ medium, each of the single, as well as the double and the triple low affinity $P_i$ transporter mutants, were more resistant to selenite than the wild-type strain or mutants of the high affinity transport system. Increased selenite resistance of the low affinity phosphate transporter mutants has been observed previously (8). The higher resistance to selenite of such mutants was ascribed to an overexpression of Pho84p. However, the mechanism by which expression of Pho84p led to selenite resistance remained unknown. The results obtained in this study provide a good explanation for this behavior. Phosphate uptake measurements in the single low affinity $P_i$ transporter mutant Δ*pho87* confirm that, in high $P_i$ conditions, Pho84p is overexpressed, and the low affinity transport system is concomitantly down-regulated. Additionally, we show that inhibition by phosphate of Pho84p-mediated selenite transport is much more effective than that of the low affinity transport system. As an example, with a simple calculation using the selenite and phosphate kinetic constants determined here, we find that in the presence of 5 mM selenite, the addition of 7.3 mM $P_i$ (as in the high $P_i$ medium) inhibits by more than 150-fold the uptake of selenite by Pho84p. In the same conditions, the uptake of selenite by the low affinity transporters is reduced by only 50%. Therefore, reduced uptake of selenite by cells expressing Pho84p, as compared with cells

using the low affinity $P_i$ transporters, is responsible for the paradoxical resistance of these strains to selenite, in high $P_i$ conditions.

Competitive inhibition of $SeO_3^{2-}$ uptake by phosphate has long been documented in various plant species (7, 33), in the green alga *Chlamydomonas reinhardtii* (34), and in the yeast *Candida albicans* (35). Thus, selenite uptake by the phosphate transport pathway could be a general mechanism, at least in plants, algae, and fungi.

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## REFERENCES

1. Rayman, M. P. (2000) *Lancet* **356,** 233–241
2. Cherest, H., Davidian, J. C., Thomas, D., Benes, V., Ansorge, W., and Surdin-Kerjan, Y. (1997) *Genetics* **145,** 627–635
3. Sors, T. G., Ellis, D. R., and Salt, D. E. (2005) *Photosynth. Res.* **86,** 373–389
4. Lindblow-Kull, C., Kull, F. J., and Shrift, A. (1985) *J. Bacteriol.* **163,** 1267–1269
5. Turner, R. J., Weiner, J. H., and Taylor, D. E. (1998) *Biometals* **11,** 223–227
6. Gharieb, M. M., and Gadd, G. M. (2004) *Mycol. Res.* **108,** 1415–1422
7. Li, H. F., McGrath, S. P., and Zhao, F. J. (2008) *New Phytol.* **178,** 92–102
8. Pinson, B., Merle, M., Franconi, J. M., and Daignan-Fornier, B. (2004) *J. Biol. Chem.* **279,** 35273–35280
9. Wykoff, D. D., and O'Shea, E. K. (2001) *Genetics* **159,** 1491–1499
10. Auesukaree, C., Homma, T., Kaneko, Y., and Harashima, S. (2003) *Biochem. Biophys. Res. Commun.* **306,** 843–850
11. Bun-Ya, M., Nishimura, M., Harashima, S., and Oshima, Y. (1991) *Mol. Cell. Biol.* **11,** 3229–3238
12. Martinez, P., Zvyagilskaya, R., Allard, P., and Persson, B. L. (1998) *J. Bacteriol.* **180,** 2253–2256
13. Zvyagilskaya, R. A., Lundh, F., Samyn, D., Pattison-Granberg, J., Mouillon, J. M., Popova, Y., Thevelein, J. M., and Persson, B. L. (2008) *FEMS Yeast Res.* **8,** 685–696
14. Oshima, Y. (1997) *Genes Genet. Syst.* **72,** 323–334
15. Ogawa, N., DeRisi, J., and Brown, P. O. (2000) *Mol. Biol. Cell* **11,** 4309–4321
16. Persson, B. L., Lagerstedt, J. O., Pratt, J. R., Pattison-Granberg, J., Lundh, K., Shokrollahzadeh, S., and Lundh, F. (2003) *Curr. Genet.* **43,** 225–244
17. Persson, B. L., Petersson, J., Fristedt, U., Weinander, R., Berhe, A., and Pattison, J. (1999) *Biochim. Biophys. Acta* **1422,** 255–272
18. Wykoff, D. D., Rizvi, A. H., Raser, J. M., Margolin, B., and O'Shea, E. K. (2007) *Mol. Cell* **27,** 1005–1013
19. Hürlimann, H. C., Pinson, B., Stadler-Waibel, M., Zeeman, S. C., and Freimoser, F. M. (2009) *EMBO Rep.* **10,** 1003–1008
20. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) *Nucleic Acids Res.* **21,** 3329–3330
21. Bisson, L. F., and Thorner, J. (1982) *Genetics* **102,** 341–359
22. Dardel, F. (1994) *Comput. Appl. Biosci.* **10,** 273–275
23. Burns, D. J., and Tucker, S. A. (1977) *Eur. J. Biochem.* **81,** 45–52
24. Letavayová, L., Vlasáková, D., Spallholz, J. E., Brozmanová, J., and Chovanec, M. (2008) *Mutat. Res.* **638,** 1–10
25. Pinson, B., Sagot, I., and Daignan-Fornier, B. (2000) *Mol. Microbiol.* **36,** 679–687
26. Tarze, A., Dauplais, M., Grigoras, I., Lazard, M., Ha-Duong, N. T., Barbier, F., Blanquet, S., and Plateau, P. (2007) *J. Biol. Chem.* **282,** 8759–8767
27. Ganyc, D., and Self, W. T. (2008) *FEBS Lett.* **582,** 299–304
28. Hürlimann, H. C., Stadler-Waibel, M., Werner, T. P., and Freimoser, F. M. (2007) *Mol. Biol. Cell* **18,** 4438–4445
29. Cost, G. J., and Boeke, J. D. (1996) *Yeast* **12,** 939–941
30. Rothstein, A. (1963) *J. Gen. Physiol.* **46,** 1075–1085
31. Rosen, B. P., and Liu, Z. (2009) *Environ. Int.* **35,** 512–515
32. Pereira, Y., Lagniel, G., Godat, E., Baudouin-Cornu, P., Junot, C., and Labarre, J. (2008) *Toxicol. Sci.* **106,** 400–412
33. Hopper, J. L., and Parker, D. R. (1999) *Plant Soil* **210,** 199–207
34. Riedel, G. F., and Sanders, J. G. (1996) *Environ. Toxicol. Chem.* **15,** 1577–1583
35. Falcone, G., and Nickerson, W. J. (1963) *J. Bacteriol.* **85,** 754–762